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DNA damage induced by nitric oxide during ionizing radiation is enhanced at replication

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ABSTRACT

Nitric oxide (•NO) is a very effective radiosensitizer of hypoxic mammalian cells, at least as efficient as oxygen in enhancing cell death *in vitro*. •NO may induce cell death through the formation of base lesions which are difficult to repair, and if they occur within complex clustered damage common to ionizing radiation, they may lead to replication-induced DNA strand breaks. It has previously been shown that 8-azaguanine and xanthine result from the reaction of guanine radicals with nitric oxide. We have now shown that adenine radicals also react with •NO to form hypoxanthine and 8-azaadenine. Cells irradiated in exponential growth in the presence of •NO are twice as radiosensitive compared to those irradiated in anoxia alone, whereas confluent cells are less radiosensitive to •NO. In addition, the numbers of DNA double strand breaks observed as γH2AX staining following radiosensitization by •NO, are higher in exponential cells than in confluent cells. DNA damage, detected as 53BP1 foci, is also higher in HF-19 cells expressing Cyclin A, a marker for cells in S and G2 phases of the cell cycle, following radiosensitization by •NO. RAD51 foci are highest in V79-4 cells irradiated in the presence of •NO compared to in anoxia, 24 h after radiolysis. This work presents evidence that radiosensitization of cells by •NO is in part through the formation of specific DNA damage, difficult to repair, which in dividing cells may induce the formation of stalled replication forks and as a consequence replication-induced DNA strand breaks which may lead to cell death.

Introduction

Radiotherapy is a common treatment regime for cancer; however survival is strongly correlated with tumour oxygenation as hypoxic cells are more difficult to kill. Ionizing radiation induces DNA damage in particular by reaction of the bases [1] with radiolytically formed hydroxyl radicals (·OH) to form adducts with the bases. Oxygen increases radiosensitivity, typically by a factor of 2–3, in part by reacting with these base radicals to form long-lived peroxyl radicals, which may interact with the adjoining sugar, and abstract a proton from the sugar residue forming a single strand break (SSB) [2]. In addition SSB are formed by the direct reaction of ·OH with the sugar moiety [3]. However, double strand breaks (DSB) are thought to be the main contributor to the lethal DNA damaging cellular effects. These are formed directly from ionizing radiation or through reaction with ·OH. Additionally SSB are generally repaired within one hour but when base modifications occur close to sites of SSB, repair can be difficult [4]. These clustered damage sites account for ~30% of damage induced by low linear energy transfer (LET) radiation [5] and if the SSBs within a clustered site encounter a replication fork, they may result in replication-induced DNA DSB [6,7] (Fig. 1).

For over 60 years the role of •NO as a radiosensitizer of mammalian cells has been investigated, for example [8–15]. However, the mechanisms of its action still remain largely unknown (see [16] for a recent review). One proposal is that •NO may react with DNA radicals formed by ionizing radiation [11,13,17–19] and in doing so form modified bases which may induce cytotoxic DNA damage. Indeed the rate of reaction of •NO with 2′-deoxyguanosine monophosphate (dGMP) hydroxyl radical adducts is of the order of $10^9\ M^{-1}\ s^{-1}$ (pH 5.5, [13]), with kinetics very similar to those of oxygen [20], which has long been established as a radiosensitizer of mammalian cells [21]. In comparison to the reaction with O_2, the reaction of •NO with a free radical would generate a non-radical species and thus prevent further chain reactions, which occur with peroxyl radical products from equivalent reactions with O_2.

The identification of potential products formed in the reaction of DNA base radicals with •NO is necessary. Early studies have

Abbreviations: •NO, nitric oxide; ·OH, hydroxyl radical; dA, 2′-deoxyadenosine; 8oxoA, 8-oxoadenine; FaPyA, 4,6-diamino-5-formamidopyrimidine; HX, hypoxanthine; 8azaA, 8-azaadenine; dGMP, 2′-deoxyguanosine monophosphate; 8azaG, 8-azaguanine; SSB, single strand break; DSB, double strand break; LET, linear energy transfer; AP, apurinic/apyrimidic.

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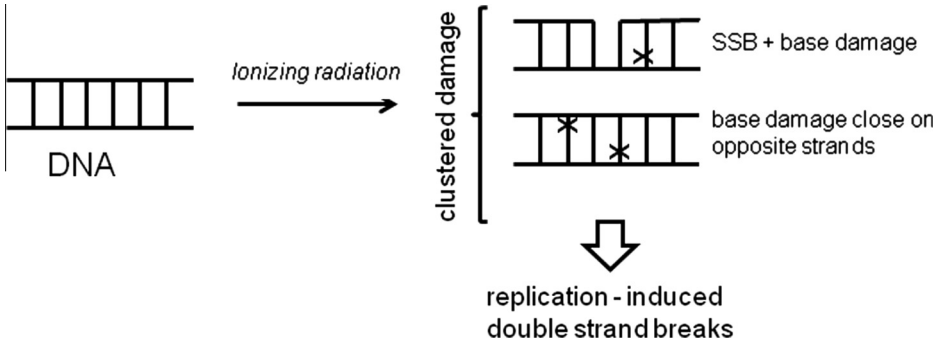


Fig. 1. Schematic of formation of clustered base damage in DNA following ionizing radiation which may lead to replication-induced double strand break.

proposed the formation of a nitroso adduct of uracil from the reaction of •NO with uracil-(·OH) [13]. In addition ·OH-adducts of guanine/dGMP react with •NO to form xanthine and 8-azaguanine (8azaG) modifications [19]. Studies with other DNA bases have not been carried out. A full understanding of the chemistry of reactions of •NO with other bases and nucleotides would be beneficial to gain a better understanding of the role by which •NO acts as a radiosensitizer.

Formation of base modifications, as a result of reactions with •NO, may be difficult to repair by conventional base excision repair, as they may not be recognized by cellular endonucleases, although this needs to be established. Previous studies measured the enhanced formation of γH2AX staining, as a marker of DSB, in hamster fibroblast cells and human breast cancer cells [13] and human prostate cancer cells [14] following radiosensitization by •NO. In V79-4 hamster fibroblasts following radiolysis in the presence of •NO it was found that the maximum number of DSB detected as γH2AX foci were formed at times >30 min when DSB are conventionally detected and at numbers 2-fold higher than those induced in anoxia alone [19]. The results suggested that the damage induced by •NO may be slow to repair. In addition, if these lesions are formed in clustered damage sites together with SSB as shown in Fig. 1 then subsequently formed DSB may arise during replication.

In this study we have investigated further if radiation-induced damage by •NO results in the formation of enhanced levels of replication-induced DSB. We also discuss preliminary investigations into changes which occur to adenine during γ-radiolysis in the presence of •NO under hypoxia and their potential role in replication-induced DSB.

Materials and methods

Materials

Dipotassium orthophosphate, potassium dihydrogen orthophosphate, sodium citrate and methanol (LC–MS grade) were obtained from Fisher, UK. 8-Azaadenine (8azaA) was obtained from MP Biomedicals, UK. DNA bases, nucleotides and nucleosides, bovine serum albumin (BSA), fish skin gelatin (FSG), paraformaldehyde (PFA), propidium iodide (PI), RNAase and cell culture solutions were obtained from Sigma–Aldrich (Poole, UK). Foetal calf serum (FCS) was obtained from Biosera, East Sussex, UK, phosphate buffered saline (PBS) tablets from Oxoid, UK and dissolved 1 tablet/100 ml. Anti phospho-histone H2AX mouse monoclonal IgG1 was obtained from Merck Millipore, USA. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 633 goat anti-rabbit IgG were obtained from Invitrogen, UK. Anti-phospho 53BP1 rabbit monoclonal antibody was obtainedfrom AbCam, UK and mouse anti-RAD51 was obtained from GeneTex, source Bioscience UK. Vectashield and anti-Cyclin A mouse monoclonal antibody were obtained from Vector Laboratories, UK. N_2O and N_2 were supplied by BOC and 1% •NO, balanced N_2 and 1% O_2, balanced N_2 from BOC special gases (Guildford, UK).

Generation of hydroxyl radicals by γ-irradiation

Radiolysis of water generates near equal amounts of oxidizing radicals (·OH) and reducing electrons (e^-_{aq}) along with low yields of protons (H^+), H·, H_2O_2 and H_2. Saturating solutions with N_2O before irradiation allows for e^-_{aq} generated through the ionization of water to be converted into ·OH (Eq. (1)) increasing the ·OH yield to ~0.6 μM Gy^{-1}. This also ensures that only the chemistry of ·OH is studied.

$$e^-_{aq} + N_2O + H^+ \rightarrow {}^{\bullet}OH + N_2 \quad (1)$$

Irradiations were carried out in a Caesium-137 GSR D1 irradiator (Gamma-service Medical GmbH, Leipzig, Germany) at dose rates confirmed by Super Fricke dosimetry [22].

Nitric oxide handling

•NO gas was handled as described previously [13]. Anaerobic conditions were maintained throughout the experimental procedure to prevent the formation of $^{\bullet}NO_2$ from reaction of •NO with O_2. Saturated solutions of 1% •NO/99 % N_2 in PBS are ~18 μM •NO at 25 °C [23] and 0.1% •NO is ~1.8 μM When the concentration of •NO required was <1% the gas was mixed with N_2O using a flow mixer and flow into the solutions was continued throughout the irradiation time, through ports in the side of the irradiator using PEEK tubing and stainless steel needles.

Reaction of adenine radicals with nitric oxide

Adenine (0.5 mM) was dissolved in potassium phosphate buffer (10 mM, pH 7.6) and saturated with N_2O for 10 min. Samples were then irradiated at 13 Gy/min for 2 min intervals with and without •NO (~1.8 μM) as described above. Products were analyzed by HPLC (Waters 2695, Watford, UK) equipped with a photodiode array detector (Waters 2996) and mass spectrometer (Waters micromass ZQ) and compared to commercially available standards. Chromatography used a Hichrom RPB column (3.2 × 250 mm, 5 μm) with a flow rate of 0.5 ml/min. Separation was achieved using 10 mM formic acid and methanol with a gradient of 3–10% methanol in 9 min and returning to starting conditions over 0.1 min. Mass spectrometry was used in electrospray positive or negative mode at 3 kV, with a cone voltage of 22 V.

Cell maintenance

Cells were obtained from the European Collection of Cell Cultures, Salisbury, UK. V79-4 hamster cell fibroblasts were grown in Eagle's modified medium (EMEM) containing 10% foetal calf serum (FCS), 2 mM glutamine, penicillin (100 U), streptomycin (0.1 mg/ml) and sodium bicarbonate (2.2g/l). HF-19 human foetal lung fibroblast cells were maintained as for V79-4 cells but in 15% FCS. Cells were harvested by trypsin digestion (0.25%).

Irradiation procedure of cells

Cell suspensions in PBS were de-oxygenated by bubbling slowly with N_2 or 1% O_2 ($\sim$ 13 μM) for 30 min in glass bubbling towers equipped with 25 ml glass syringes. The N_2 gas supply was then changed to 1% $^{\bullet}$NO ($\sim$18 μM$^{\bullet}$NO) where required for a further 20 min as described previously [13]. Cell suspensions were irradiated in glass capped syringes at room temperature (RT).

Clonogenic Survival

V79-4 cells were grown to confluence or harvested in exponential growth and suspended in PBS at $\sim 5 \times 10^5$ cells/ml. Small samples were fixed in 70% ethanol and stored at −20 °C for measurement of cell cycle status. Following gas saturation, cell suspensions were irradiated at 0.5 Gy/min for up to 5 Gy. ~2 ml samples were removed after each dose and placed on ice. Irradiated cells were counted (~250–750 cells) using a FACS Vantage flow cytometer equipped with a Sort Enhancement Module (Becton Dickinson, UK) into triplicate 25-cm^2 tissue culture flasks containing EMEM. Cells were grown for 7 days at 37 °C. Colonies were fixed with 75% methanol and stained with methylene blue (1% w/v in water). Surviving fraction (SF) was calculated by counting the number of colonies (>50 cells) and dividing by the number of cells plated. SF were then corrected by dividing by the plating efficiency (calculated by measuring the SF of un-irradiated but gas bubbled cells).

Measurement of cell cycle status

G1, S and G2/M phases of the cell cycle were monitored by staining cellular DNA with propidium iodide (PI). Stored samples were warmed to room temperature (RT) then spun (5 min, 250 g) to remove the ethanol. 0.5 ml PI (20 μg/ml) containing RNase (4 μg/ml) was added to each sample, mixed and incubated at 37 °C for 30 min. Analysis was carried out on a FACScan flow cytometer (Becton Dickinson, UK) using CellQuest software with 10,000 events counted. A histogram was created of PI fluorescence against number of cells and the proportion of cells in G1, S, and G2 phases calculated by a ModFit program (Verity Software House). Cells were regarded as confluent if the proportion of population in G1 was >65 %.

Measurement of γH2AX foci formation and repair using flow cytometry

V79-4 cell suspensions obtained from confluent monolayers or exponentially growing cells (15 ml PBS at 3–5 $\times 10^5$ cells/ml) were saturated with gas and irradiated (5 Gy) in sealed glass syringes. Following irradiation the cell suspensions were placed on ice in polypropylene tubes before centrifuging (5 min, 250 g, 4 °C). Pellets were resuspended in EMEM and cells were plated ($\sim 1.5 \times 10^6$ cells) into six well plates at 37 °C. Up to 24 h after plating, the cells were scraped off and spun as above. Pellets were fixed and stored as above. Fixed cells were warmed to RT then spun (250 g, 5 min) to remove the ethanol and washed with 2% FCS in PBS (0.5 ml). The cell pellet was incubated with anti-phospho histone H2AX (100 μl, 1:300 in 2% FCS/PBS) for 1 h at RT. Cells were washed as above. Secondary antibody Alexa Fluor 488 (100 μl, 1:300 in 2% FCS/PBS) was then added (1 h, RT) and kept out of light. Cells were washed once more and DNA stained with PI containing RNAse as described above. Cells were analyzed within 24 h using a FACScan flow cytometer (Becton Dickinson) running CellQuest software. 10,000 cells were counted and the results analyzed using WinMDI (http://facs.scripps.edu/software.html), gated for G1–G2 cell cycle status. The average green fluorescence, indicating γH2AX foci formation was measured from a Histogram plot of fluorescence intensity and normalized to control un-irradiated cells.

Measurement of γH2AX foci in V79-4 cells

Cells were treated as described previously [19]. Exponentially growing cells were suspended in PBS ($\sim 3 \times 10^5$/ml) and saturated with N_2 or 1% O_2 (~13 μM). Cells were then either irradiated (2 Gy at 0.5 Gy/min) or bubbled further with 1% $^{\bullet}$NO for 20 min before irradiation. Cells were then placed on ice, spun (5 min, 200 g) and re-suspended in EMEM. Cells ($\sim 6 \times 10^5$) were plated onto glass walled dishes with glass cover slip bases, incubated for 0.5–24 h at 37 °C and then washed with PBS before fixing with 3% paraformaldehyde (PFA) and stored at 4 °C before immunofluorescent staining. Cells were washed with PBS (3 × 1 ml, 5 min), lysed with 1% Triton X-100 in PBS (0.5 ml, 10 min) and washed again. Dishes were then incubated in 1% FSG/1% BSA (0.5 ml) for 1 h before incubation with primary antibody (200 μl, 1:3000) anti phospho-histone H2AX overnight at 4 °C. Cells were then washed and incubated in secondary antibody (200 μl, 1:3000) Alexa Fluor 488 goat anti-mouse for 1 h at RT. Cells were finally washed once more and mounted under glass coverslips with a drop of Vectashield containing DAPI and stored at 4 °C in the dark. Foci were measured using a Biorad MRC 600 confocal microscope equipped with a x40 oil objective and counted using ImageJ software (http://rsbweb.nih.gov/ij/).

Irradiation of cell suspensions for detection of 53BP1 foci in replicating cells

Human HF-19 cells were used in these studies as the antibody stains for Cyclin A in human cells but not hamster cells. HF-19 cells in exponential growth were suspended in PBS ($\sim 3 \times 10^5$/ml) and saturated with gases as described above. Cells were irradiated (2 Gy at 0.5 Gy/min) and then placed on ice. They were spun (200 g) and re-suspended in EMEM and plated ($\sim 6 \times 10^5$ cells) onto glass dishes for 4–24 h at 37 °C. Cells were then washed with PBS (1 ml) and fixed with 3% PFA in PBS, and stored at 4 °C before immunofluorescence staining. Cells were washed with PBS (3 × 1 ml, 5 min), lysed with 1% Triton X-100 in PBS (0.5 ml, 10 min) and washed again. Dishes were then incubated in 1% FSG/1% BSA (0.5 ml, 1 h) , washed and then incubated with primary antibodies (200 μl, 1:500 anti 53BP1 and 1:50 anti Cyclin A) overnight at 4 °C. Cells were washed again and incubated in secondary antibodies (200 μl, 1:1500 Alexa Fluor 488 goat anti-mouse and 1:3000 Alexa Fluor 633 goat anti-rabbit) for 1 h at RT. Cells were washed a final time and mounted under glass coverslips with Vectashield containing DAPI and stored at 4 °C in the dark for up to 2 days. Foci were detected using a Biorad MRC 600 confocal microscope equipped with a x40 oil objective and counted as above.

Irradiation of cell suspensions for detection of RAD51 foci

V79-4 cells in exponential growth were suspended in PBS ($\sim 3 \times 10^5$/ml) and saturated with gasses. Cells were irradiated (10 Gy at 1.6 Gy/min) and then placed on ice, spun (200 g) and

re-suspended in EMEM. Cells ($\sim 6 \times 10^5$) were plated onto glass dishes for 4–24 h at 37 °C and then washed (1 ml PBS), and fixed for 15 min in cold 3% PFA/0.1% Triton X-100, followed by washing again with PBS (1 ml, 3 × 5 min). Then they were incubated with PBS/0.3% Triton X-100 (0.5 ml, 10 min) and non-specific protein binding was blocked with 3% BSA (0.5 ml, 40 min). RAD51 foci were detected using mouse anti-RAD51 (200 μl, 1:1000) in 3% BSA, 4 °C, overnight. Cells were washed again and incubated in secondary antibody (200 μl, 1:3000 Alexa Fluor 488 goat anti-mouse) for 1 h at RT. Cells were then washed again and mounted under glass coverslips and foci measured as described above.

Results

Reaction of nitric oxide with adenine radicals

Adenine is known to react with ·OH to form mainly 4,6-diamino-5-formamidopyrimidine (FaPyA) and 8-oxoadenine (8oxoA) [3]. The presence of 0.1% •NO under anaerobic conditions at pH 7.6 significantly reduces the yield of 8oxoA (retention time 6.6 min) identified by *m/z* 152 (M + H). In addition the peak with retention time 2.6 min and *m/z* 152 (M − H), strongly suggestive of FaPyA, is also significantly reduced. However, two new products are formed (Fig. 2A). The first product (HX), with a retention time of 5.9 min, exhibits a *m/z* of 137 (M + H), 1 Da greater than adenine, and by co-elution with an authentic standard is identified as hypoxanthine (Fig. 2B). This product is also observed when adenine is treated with acidified nitrite at pH 3.7. The second product with a retention time of 7.4 min also exhibits a *m/z* of 137 (M + H). It is not observed when adenine is reacted with acidified nitrite at pH 3.7. The product is proposed to be 8-azaadenine (8azaA) by comparison with the formation of 8azaG detected in similar reactions with guanine [19]. An authentic sample of 8azaA co-elutes with the product obtained from the radiolysis studies (Fig. 2B), confirming the assignment to the radiolytic production of 8azaA. γ-Radiolysis of N_2O-saturated solutions of adenine (0.5 mM) in 10 mM phosphate at pH 7.4 in the presence of ~1.8 μM •NO at a dose rate of 13 Gy/min results in the formation of HX and 8azaA with radiation chemical yields (*G* values) of 0.034 ± 0.001 μM/Gy and 0.100 ± 0.001 μM/Gy, respectively (Fig. 2D).

Effect of cell cycle on radiosensitization efficiency by •NO

Modification of DNA by radiation in the presence of •NO under anaerobic conditions results in base damage and if not repaired efficiently before a replication fork is encountered, a replication-induced DSB may be formed. By studying the formation of DSB, detected as γH2AX formation, in cells irradiated either in exponential growth or in a confluent state we have addressed whether replication-induced DSB could be formed and as a consequence may contribute to cytotoxicty.

Cell survival

In confluent V79-4 cells in the presence of ~18 μM •NO cell survival is reduced in the dose range studied up to 5 Gy. The survival enhancement ratio (SER) at 0.4 SF was determined to be 1.4 (Fig. 3A). In replicating cells the SER is higher with a value of 2.0 at 0.4 SF (Fig. 3B). Although radiosensitization in the presence of •NO under anoxia is similar in both growth conditions, in the absence of •NO, replicating cells are more resistant to radiation than their non-replicating confluent counterparts as has been previously shown [24].

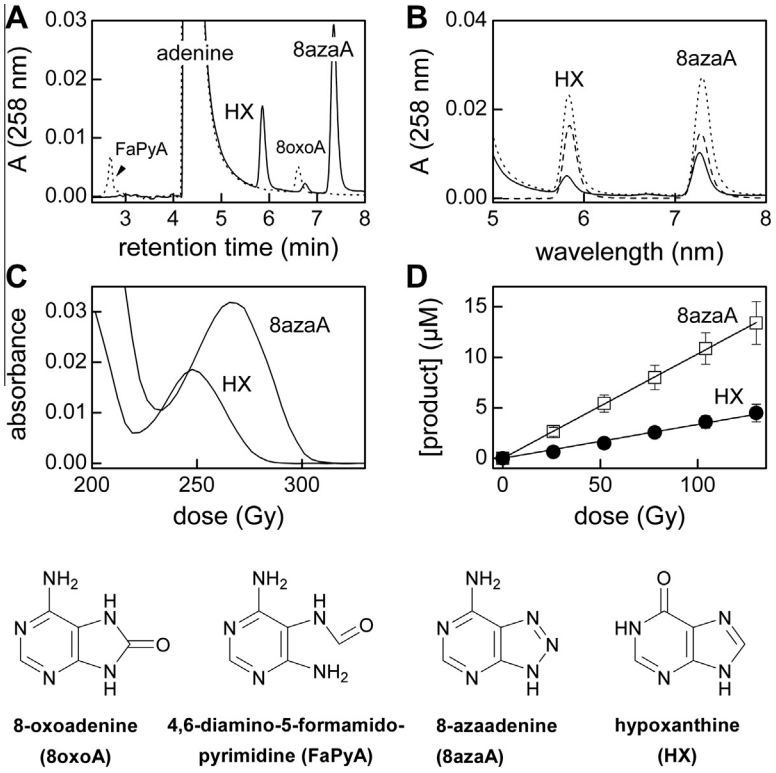


Fig. 2. (A) Chromatogram monitoring at 258 nm showing oxidation of adenine (0.5 mM) at pH 7.6 by ·OH after 130 Gy in the absence of (dotted line) or in the presence of ~1.8 μM ·NO (solid line); (B) HPLC chromatogram showing the products (diluted 1 in 2) from the oxidation of adenine (0.5 mM) at pH 7.6 by ·OH after 130 Gy in the presence of ~1.8 μM ·NO/~22.5 mM N_2O (solid line); authentic samples of hypoxanthine (HX) and 8-azaadenine (8azaA) (dashed line); products from 130 Gy sample spiked with authentic samples of HX and 8azaA (dotted line); (C) UV–visible spectra of HX and 8azaA; (D) yield of HX and 8azaA on dose following the reactions as described above. Results show the mean and SD of 4 independent experiments.

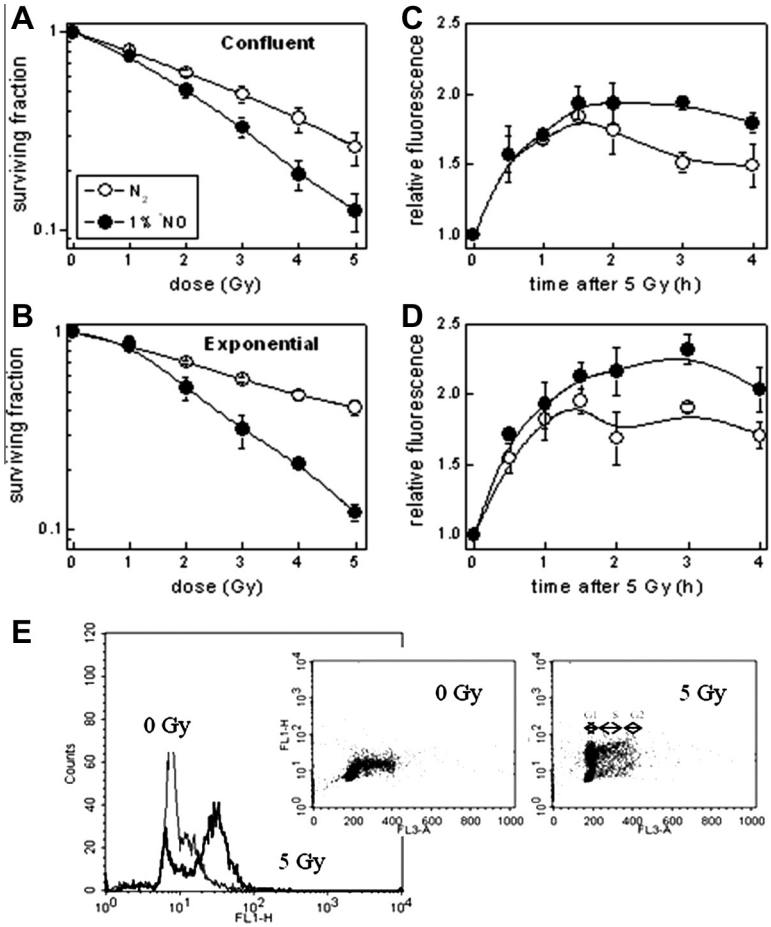


Fig. 3. Cell survival of V79-4 cells with dose following γ-radiation in N_2 (○) or ~18 μM $^{\bullet}NO/N_2$ (●) saturated cell suspensions in (A) confluent and (B) exponential growth cells; Effect of time on the formation of γH2AX foci in (C) confluent and (D) exponential growth cells. Results represent the mean and s.e.m. of at least three independent experiments; (E) flow cytometry histogram and dot plots gated for G1–G2 status showing the increase in fluorescence with radiation in exponential cells 2 h following γ-irradiation with 0 or 5 Gy when saturated with ~18 μM $^{\bullet}NO/N_2$.

Formation of γH2AX foci as markers of DNA double strand breaks

V79-4 cells irradiated in exponential growth express a slightly higher level of γH2AX staining measured by flow cytometry than seen with confluent cells within 1 h following irradiation with 5 Gy (Fig. 3E). The presence of nitric oxide enhances the level of γH2AX especially at times >90 min (Fig. 3C and D). Cells exposed to nitric oxide express maximum γH2AX staining ~3 h after irradiation compared to cells irradiated in anoxia alone, which show maximum staining after ~1 h (Fig. 3C and D).

Exponentially growing V79-4 cells irradiated in the presence of 1% O_2~13 μM) are radiosensitized to a similar extent as that by 1% $^{\bullet}NO$ (~18 μM) (Fig. 4B). In contrast, differences were seen between these two conditions in the level of γH2AX foci/cell. The loss of γH2AX foci with time indicate that DSB are repaired more efficiently in 1% O_2 (Fig. 4A). In contrast significant levels of γH2AX foci are present at times >2 h in the presence of 1% $^{\bullet}NO$ and persist at least for up to ~6 h as was seen previously [19]. The persistence of DSB is consistent with formation of replicative DSB.

Formation of 53BP1 foci in replicating cells

HF-19 cells irradiated in exponential growth in the presence of ~18 μM $^{\bullet}NO$ balanced N_2 show ~2-fold increase in the number of 53BP1 foci per cell 4 h after plating following 2 Gy γ-radiation compared with cells irradiated in anoxia alone (Fig. 5A and B). The majority of foci resulting in an ~2-fold increase were observed in cells also expressing Cyclin A, a marker of cells in S and G2 phases of the cell cycle (Fig. 5C).

Formation of RAD51 foci as markers of stalled replication forks

V79-4 cells irradiated (10 Gy) in exponential growth in the presence of ~18 μM $^{\bullet}NO$ balanced N_2 show ~2-fold more RAD51 foci/positively stained cell 24 h after irradiation than cells irradiated in anoxia alone (Fig. 6A). However, little difference is seen between the two conditions at earlier times. The observation of RAD51 foci at longer times and a higher proportion of cells expressing RAD51 foci (Fig. 6B) indicate that $^{\bullet}NO$ has induced complex damage which is repaired more slowly by homologous recombination, or that replication forks are stalled when encountering clustered damage containing base modifications formed in the presence of $^{\bullet}NO$.

Discusssion

Nitric oxide is an efficient radiosensitizer of hypoxic cells and DNA damage may be involved in the mechanisms by which $^{\bullet}NO$ enhances cell toxicity. DSB are enhanced when $^{\bullet}NO$ is present during radiolysis [13,14], although some of these DSB may represent replication induced DSB as discussed previously [19]. Nitric oxide may elicit DNA damage because of the formation of unprocessed apurinic/apyrimidic (AP)-sites which result in DSB [25]. In plasmid DNA, $^{\bullet}NO$ protects against the formation of

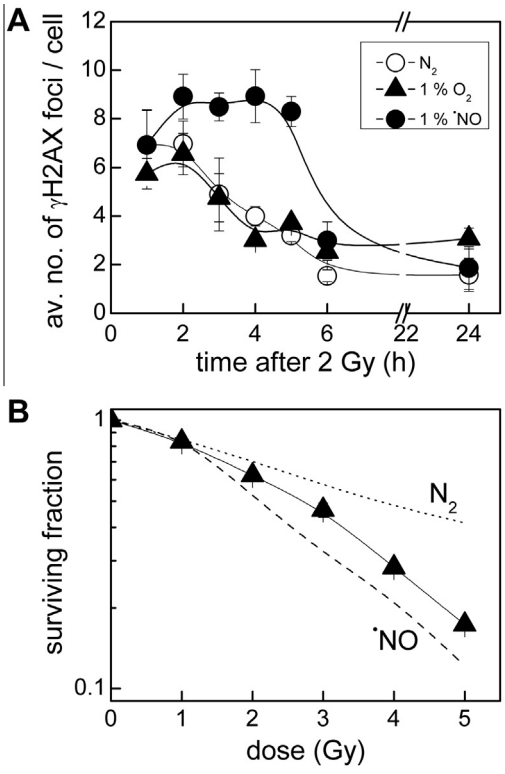


Fig. 4. (A) Effect of time following plating in EMEM ~15 min after γ-radiation (2 Gy) on the number of γH2AX foci per cell in exponential V79-4 cells in N_2 (○), ~18 μM •NO/N_2 (●) or ~13 μM O_2 /N_2(▲) saturated cell suspensions in exponential growth; (B) clonogenic survival curves following irradiation in 1% O_2. The survival data for irradiations carried out in N_2 and •NO curves are taken from Fig. 4A and B. Results represent the mean and s.e.m. of at least three independent experiments.

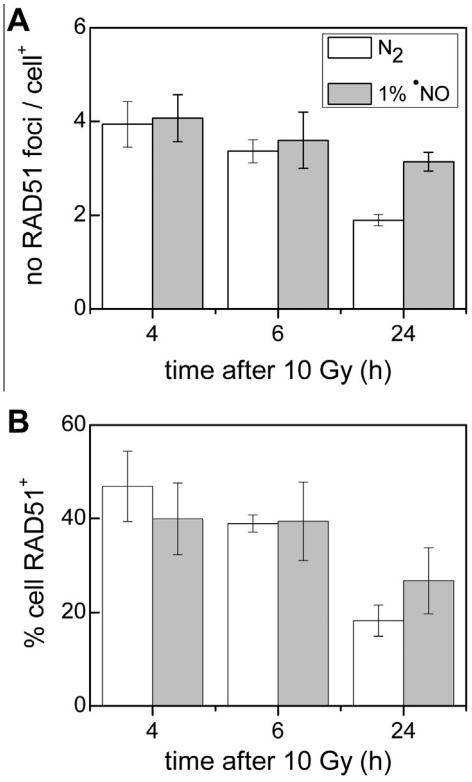


Fig. 6. Effect of time after plating in EMEM ~15 min following γ-radiolysis (10 Gy) of suspensions of V79-4 cells saturated with N_2 or 1 % •NO (~18 μM)/N_2 on; (A) the number of RAD51 foci per positive RAD51 cells and (B) the percentage of cells expressing RAD51 foci. Results represent the mean and s.e.m. of at least three independent experiments.

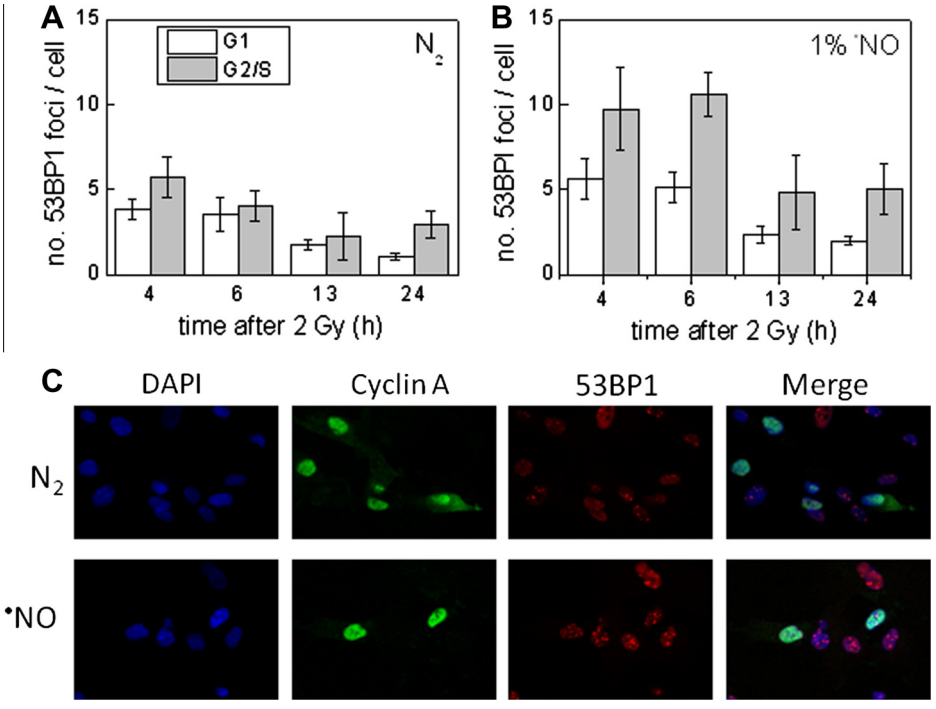
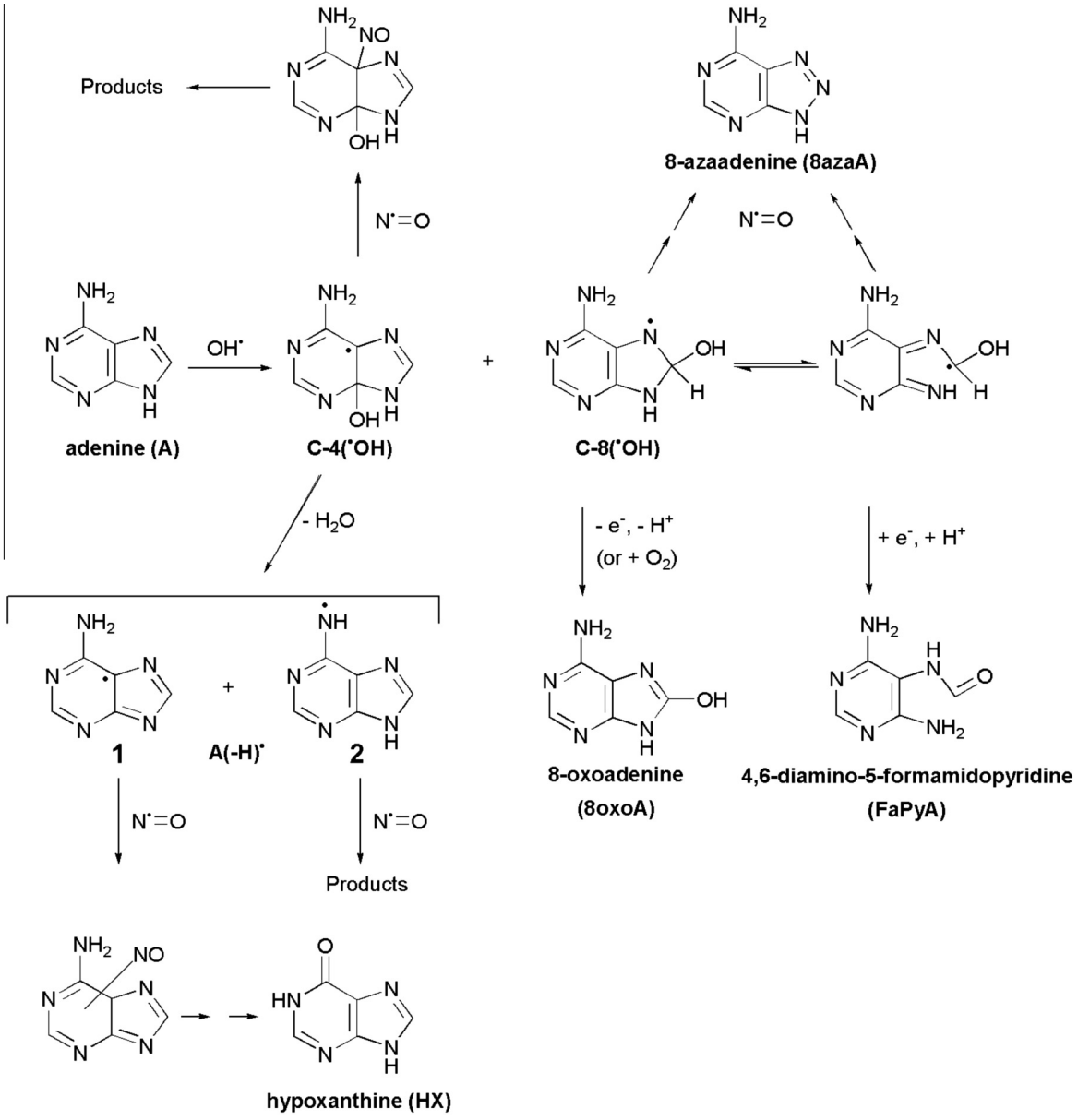


Fig. 5. Effect of time on the number of 53BP1 foci induced in G1 or S/G2 (Cyclin A positive) phases of the cell cycle following plating ~15 min after γ-radiolysis (2 Gy) of HF-19 cells in (A) N_2 saturated cell suspensions or (B) •NO (~18 μM)/N_2 saturated cell suspensions. Results represent the mean and s.e.m. of at least three independent experiments; (C) Confocal images (x40) 6 h following radiolysis. DAPI and Cyclin A staining indicate the nucleus and cells in S/G2 phase respectively.

radiation-induced SSB in anoxia, however digestion with Fpg glycosylase, which recognizes purine modifications, increases the number of enzyme-induced SSB and DSB [19].

Use of radiation chemistry techniques has helped us to understand how the presence of •NO in hypoxic cells during radiotherapy could form damaged DNA bases, possibly in clusters, which may cause toxicity through the induction of strand breaks following replication or during repair processes. We have previously shown that radiation-induced ·OH- adducts of guanine/dGMP react with •NO to form xanthine and 8azaG modifications [19], the latter being a cytotoxin which has been investigated for cancer treatment [26,27]. Modifications to the other DNA bases will also occur, although the majority of studies into DNA damage by ionizing radiation tend to focus on guanine as it is the most easily oxidized of the bases [28]. In this work we have extended our studies to adenine.

Adenine reacts with ·OH to form mainly two adducts positioned at C-4 (~50%) and C-8 (~37%) [29]. Products of C-8(·OH) are 8oxoA and the ring-opened FaPyA (Scheme 1). The former predominates in oxygenated systems (O_2 > 30 μM) [30]. We propose that C-8-·OH adducts of adenine react with •NO, significantly reducing the yields of 8oxoA and FaPyA but resulting in the formation of a new product 8azaA (Scheme 1, Fig. 2) in a similar mechanism to that proposed with guanine [19]. However, with 2́-deoxyadenosine (dA), the formation of 2́-deoxy-8-azaadenosine is very low if formed at all. Similarly, when dA is oxidized by ·OH in the presence of •NO, the formation of 2́-deoxyinosine (the nucleoside equivalent of HX) is also very low, if formed at all. Based on this observation we propose that HX is formed from the reaction of •NO with either the C-4(·OH) adduct of adenine or preferentially with radical 1 resulting from dehydration of the C-4(·OH) adduct (k = 1.3×10^5 s^{-1}[31]) (Scheme 1). The presence of the deoxyribose sugar on N-9 of adenine seems to influence the subsequent chemistry of product formation. It is known [32] that the ribose sugar on N-9 of adenine causes the rate of dehydration of the equivalent C-4(·OH) radical of dA to be lower by a factor of ~4



Scheme 1. Proposed mechanisms for the reaction of nitric oxide with OH adducts of adenine. For simplicity, only one of the possible mesomeric forms of each radical center is shown.

($k = 2.9 \times 10^4$ s^{-1}[33]). Scheme 1 outlines the potential pathways to formation of HX and 8azaA. Further work is necessary to elucidate fully the chemistry and products formed following •NO-reaction with the ·OH-adducts of dA/dAMP and the role of substitution at N-9.

The chemistry of the reaction of pyrimidines with ·OH is well understood although they are less easily oxidized than the purines. Uracil-·OH adducts react with •NO with a rate constant of 3.7×10^9 dm^{-3} mol^{-1} s^{-1}[13]. A product suggestive of an –OH and –NO adduct was observed but its identity has not been confirmed. Reaction of •NO with ·OH adducts of thymine and cytosine form different products to those observed in the absence of •NO (L K Folkes personal communication) but their identification has so far not been possible as more stringent analytic methods are required to confirm whether •NO- adducts are indeed formed. Interestingly with the nucleosides/nucleotides of adenine, thymine and cytosine the modifications which are observed with the equivalent free bases are not clearly replicated when the ribose sugar is present, in contrast to the findings with guanine nucleotide [19].

The steady state γ-radiolysis carried out in anoxia has enabled us to investigate the reactions of base radicals with •NO rather than reactions of bases with $•NO_2$ which may form from •NO in the presence of O_2. In tumours however, anoxic regions would be unlikely to contain viable cells and it is the hypoxic regions which are particularly difficult to kill. Hypoxia (O_2 < 1%) is common to many tumours [34] and cells in these regions are radioresistant, as O_2 is an efficient radiosensitizer. •NO is also an efficient hypoxic cell radiosensitizer, and is at least as good as O_2[13]. Indeed a mixture of 800 ppm O_2, 80 ppm •NO showed radiosensitivity in V79-379A cells equivalent to •NO alone [13]. In addition, the presence of •NO in hypoxic tissue may also lead to the formation of –OONO derivatives of DNA bases from the reaction with base peroxyl radicals. These products have been proposed to form additional radicals in secondary reactions [35].

The differences in the products formed from reaction of ·OH adducts of the bases with O_2 or •NO may have a marked effect on the formation of strand breaks in DNA. Plasmid DNA irradiated in the presence of •NO exhibits ~3-fold less SSB than that irradiated in anoxia [19]. In comparison O_2 increases the formation of strand breaks through the intermediate formation of peroxyl radicals in cells [3]. However, we have shown that formation of DSB detected as γH2AX foci, in cells irradiated in 1% O_2 are lower than those induced in 1% •NO 2–5 h post-irradiation (Fig. 4A). This suggests that the damage induced by •NO persists for longer times following irradiation than that induced in 1% O_2. The more persistent damage detected as γH2AX foci may be indicative of replicative DSB as a consequence of DNA damage meeting a replication fork in S-phase. In addition cells irradiated in exponential growth are more sensitive and exhibit more γH2AX foci than those cells irradiated in confluency (Fig. 3), consistent with the increased radiosensitivity of cells in G2/M phase by •NO as previously reported [36]. Colon cancer cells are also most sensitive to •NO in the absence of radiation in G2-M phase [37]. We have shown that irradiated HF-19 cells expressing Cyclin A, a marker of S/G2 phases of the cell cycle, contain more 53BP1 foci in S-phase cells than cells in G1, particularly in cells irradiated in the presence of •NO (Fig. 5). RAD51, a protein involved in homologous recombination (HR), repairs DSB during S and G2 phases of the cell cycle and also replication-induced DSB from stalled replication forks [7,38,39]. Since the numbers of RAD51 foci are higher 24 h after γ-radiolysis of V79-4 cells in the presence of •NO than those irradiated in anoxia alone (Fig. 6), the enhancement in γH2AX and RAD51 foci following ionizing radiation in the presence of •NO suggests that •NO- induced DNA damage, which is more difficult to repair compared to damage induced in the presence of O_2, leads to replication-induced DSBs during S-phase through encountering a replication fork.

Conclusions

Nitric oxide is able to radiosensitize cells under hypoxia, particularly those in S-phase as a consequence of stalled replication. DSB are generated in excess of the number formed in the presence of similar concentrations of O_2. Tumours which express high levels of •NO expressed in inflammatory conditions may also be particularly susceptible to radiosensitization. In addition, the DNA base modifications identified in these studies may also be formed in these conditions where oxidative and nitrosative stress is high. The use of nitric oxide for increasing radiotherapy effect is promising and delivery of the gas to hypoxic tissue close to the time of radiotherapy without sustaining levels post radiotherapy may offer the greatest opportunity for killing tumours. Development of drugs which release •NO only in hypoxia would be beneficial and offer further targeting to these less radiosensitive regions of tumours.

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